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TITLE: "Evaluation of Human Adipose Tissue Stromal Heterogeneity in Metabolic Disease Using Single Cell RNA-Seq"

PRINCIPAL INVESTIGATOR: Dr. Linus Tzu-Yen Tsai, Ph.D., M.D.

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center, Boston
Boston, MA 02215-5400

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Linus Tzu-Yen Tsai

E-Mail: ltsai@bidmc.harvard.edu

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BETH ISRAEL DEACONESS MEDICAL
CENTER, 330 BROOKLINE AVE
BOSTON MA 02215-5400

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14. ABSTRACT

We have developed a robust protocol to generate single cell transcriptional profiles from subcutaneous adipose tissue samples of both human and mouse subjects using Drop-seq, a recently developed, cost-efficient method of highly parallel genome-wide expression profiling using nanoliter droplets. We have collected subcutaneous adipose tissue samples from >15 human subjects and generated transcriptional profiles for over 46,000 individual cells. Additionally, we have generated profiles from ~10,000 cells from mouse subcutaneous and epididymal depots. In both data sets, our analyses demonstrate expression profiles can be used to cluster individual cells into distinct cell types in an unbiased fashioned. We identify most cell types known to be contained within adipose tissue SVF and have uncovered a number of cell types and subtypes that have not previously been described. We determine novel markers for many cell types with higher specificity than current markers and characterize many species and depot-specific cell types and markers. These data provide a comprehensive transcriptional atlas of subcutaneous adipose tissue cell types that will provide molecular handles to understanding and manipulating each cell type's function. These results are hypothesis-generating, and provide the foundation for future studies that will 1) define functional roles for individual genes and cell types in development of obesity and insulin resistance and 2) examine novel targets against which we can design therapies to target specific pathogenic or or health-promoting cell types.

15. SUBJECT TERMS

Obesity, Type 2 Diabetes Mellitus, Insulin resistance, Adipose, Stromal vascular fraction

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INTRODUCTION:

The overall goal of this proposal is to determine how individual cell types within human adipose tissue interact to regulate adipose tissue physiology. Specifically, we have developed the molecular and analytical tools to identify and classify the identity and function of individual cell types within the adipose tissue stromal vascular fraction (SVF) in an unbiased fashion using single-cell transcriptional profiling. By comparing adipose tissue samples from a range of healthy and diseased individuals, we have begun to explore how individual cell types work in concert to maintain adipose tissue health, and how this cellular network is compromised with chronic overnutrition (obesity).

KEYWORDS:
Obesity, Diabetes, Insulin Resistance, Adipose, Adipocytes, Stromal Vascular Fraction, Single-cell RNA-seq, Transcriptional profiling, Drop-seq, Adipose Depot

ACCOMPLISHMENTS:

What were the major goals of the project?

Specific Aim 1: Use single-cell Single-cell RNA-seq to profile gene expression in individual SVF cells from adipose tissue across a spectrum of metabolic phenotypes	**Milestone/ Target Date (Months)**	**Completion Date**	**% completion**
Specific Aim 1: Comparing SVF from lean and obese individuals			
Milestone Achieved: HRPO/ACURO Approval	3	01/14/2016	100%
FACS initial SVF samples (2 human subjects, 2000 cells) into individual plates	3-6	04/2016	100%
Adaptation of SCRB-seq protocol to human SVF	3-6		0%
Development of Dropseq for single cell profiling	6-15	12/2016	100%
Collect initial SVF samples (5) with generation of Single-cell RNA-seq Libraries and sequence	10-18	01/2017	100%
Analyze initial 20,000 cells for overall cellular complexity and evaluate pilot to determine how many cells will be needed per sample (Monocle, Cufflinks software)	15-18	03/2017	100%
Milestone Achieved: determination of number of cells per sample required	15	12/2017	100%
Analyze initial 6000 cells for overall cellular complexity and evaluate pilot to determine how many cells will be needed per sample (Monocle, Cufflinks software)	18-30	06/2016	100%
Milestone Achieved: determination of number of cells per sample required	18-30	12/2016	100%
Collect further obese/leanSVF samples (10-20 human subjects, see proposal for discussion of targeted number)	18-30	9/2017	100%
Generate Dropseq RNA-seq Libraries and Sequence from further samples (directly above)	18-30	9/2017	100%
Milestone Achieved: Single-cell RNA-seq of SVF from target number of lean/obese individuals (see proposal for discussion of targeted number)	30	9/2017	100%
Analyze Single-cell RNA-seq data for changes in cellular complexity that occur in lean and obese groups and determine gene and cellular networks governing phenotypes (Monocle, Cufflinks, GSEA, ARACNE software)	24-30		50%
Aim1b: Comparing SVF from insulin sensitive and insulin resistant subjects			
Collect further insulin sensitive and insulin resistant SVF samples(10) with generation of Single-cell RNA-seq Libraries and sequence	18-30		50%
Milestone Achieved: Single-cell RNA-seq of SVF	30		50%

from target number of insulin sensitive and resistant individuals (see proposal for discussion of targeted number)			
Analyze Single-cell RNA-seq data for changes in cellular complexity that occur in insulin sensitive versus insulin resistant groups and determine gene and cellular networks governing phenotypes, as above	24-30		50%
Specific Aim 2: Use single-cell Single-cell RNA-seq to profile SVF from paired subcutaneous and visceral fat samples			
Aim 2a. Compare paired human SVF samples from subcutaneous and visceral depots			
Collect paired samples (12 human subjects)	18-30		10%
Generate Single-cell RNA-seq Libraries and Sequence	18-30		10%
Milestone(s) Achieved: Single-cell RNA-seq of subcutaneous versus visceral SVF from 12 (see proposal for discussion of targeted number)	30		10%
Analyze Single-cell RNA-seq data for changes in cellular complexity that occur between subcutanesous and visceral depots and determine gene and cellular networks governing phenotypes	24-30		10%
Aim 2b. Compare paired mouse SVF samples from subcutaneous and visceral depots	**NEW AIM**		
Collect paired samples from inguinal (SC) and epididymal (visceral) depots from 2 pools of Chow-Fed Mice	18-24	7/2017	100%
Collect paired samples from inguinal (SC) and epididymal (visceral) depots from 3 pools of both Chow-Fed Mice and HFD-fed mice	24-30		25%

What was accomplished under these goals?
During this intermediate phase of this grant (we applied for an extension due to slower sample accrual and switch from SCRB-seq technology to Drop-seq), we have focused on sample accrual and preliminary analyses. While continuing to optimize the cell dissociation and Drop-seq protocol to consistently and robustly generate single cell transcriptional profiles for adipose tissue, we have data from >46,000 individual cells from 15 different individuals (14 are subcutaneous samples and 1 is visceral omental) libraries. We have had some delays in sample accrual specifically for paired subcutaneous and omental samples (Aim 2) due to the primary bariatric surgeon aiding us in obtaining these samples leaving this summer to another institution. We are currently in discussion with multiple general and gynecologic surgeons at our institution to obtain these omental samples.

During this delay, we began collecting paired subcutaneous and visceral samples from mice exposed to control and high fat diet (HFD), which robustly induces obesity and insulin resistance. We pursued these cross-species comparisons for a number of practical and scientific reasons: first, our preliminary data analysis of human data show strong effects of cell type clustering that is driven by the individual the cell came from (this could result from shared genetic or epigenetic causes). Examining inbred mice allows us to look at genotype-independent determination of cell types. The ability to control environmental factors like diet, degree of fasting, anesthesia given provides a degree of epigenetic stability not obtainable with

human experiments, providing the power to identify more statistically significant differences. Lastly, given that validation experiments will be performed in mice, we felt it important to determine how well mice modeled human cell types and functions. We have thus far profiled individual SVF cells from inguinal (2,500 cells) and epididymal (7,500 cells) depots from Chow-fed diet and 2000 epididymal cells from HFD-fed mice.

Initial clustering of ~46,000 human cells (Figure 1A) shows the unbiased identification of most expected cell types (pre-adipocytes, macrophages, B cells, T cells, NK cells, endothelial cells, vascular smooth muscle cells and pericytes, as well as subtypes of some of these cell types (eg. M1, M2, and phagocytic macrophages). There are many pre-adipocyte clusters that are largely defined by the origin of individual (Figure 1B), which suggests strong genetic and epigenetic drivers of clustering. We can determine distinct marker transcripts whose expression defines cells within cluster (Figure 1C).

Figure 1: tSNE plots of Human SVF clusters. Labeled by A) cell type or B) individual C) Heatmap of individual cells for cluster marker gene expression

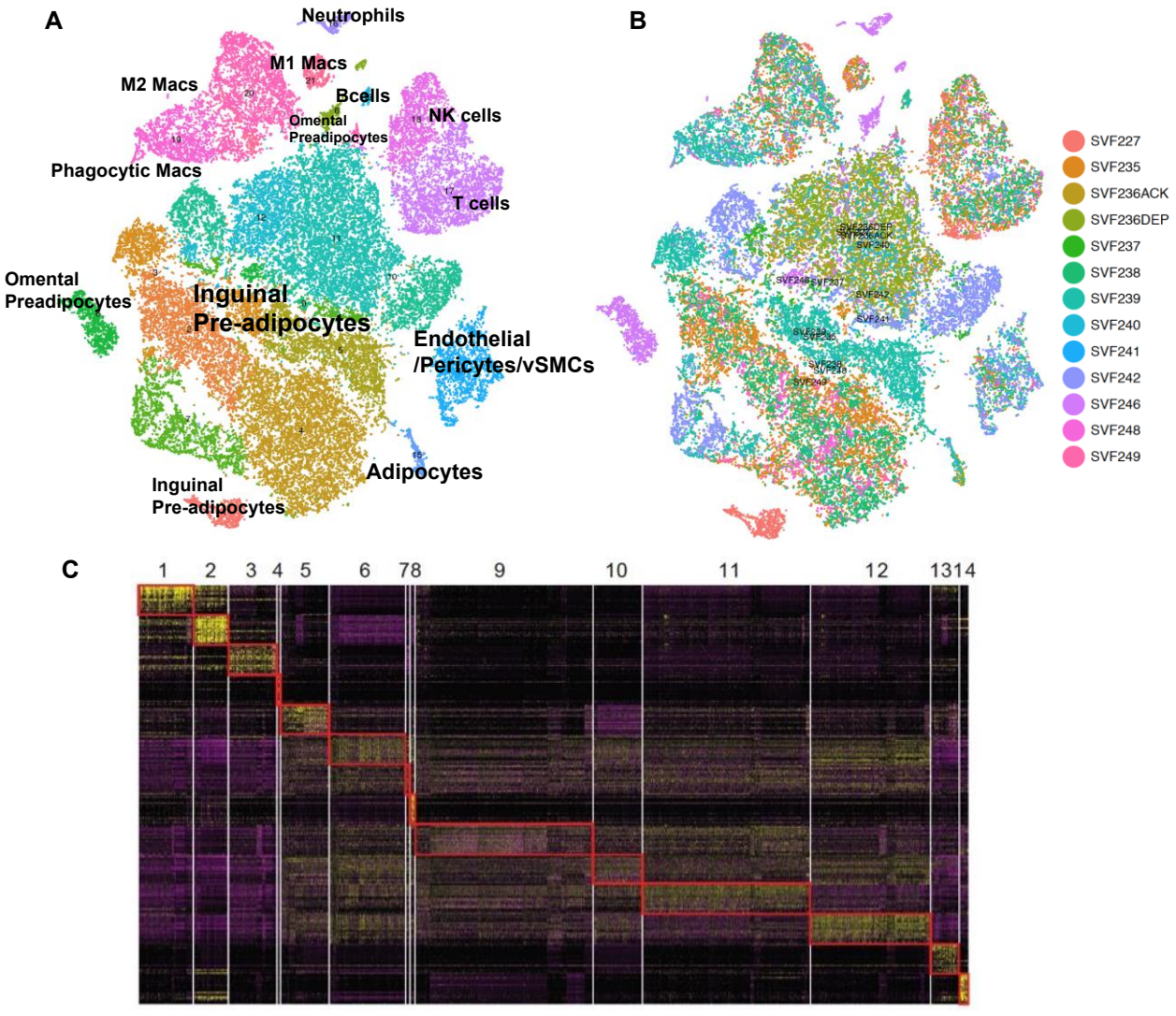
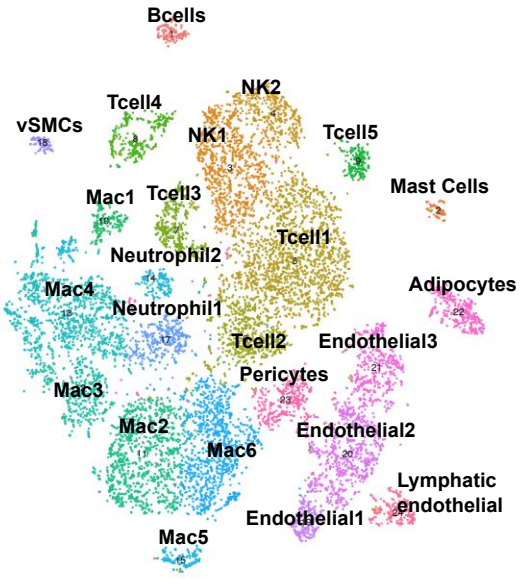


Figure 2: tSNE plots of non-preadipocyte cells from Human SVF



Further subclustering of non-preadipocyte populations allows identification of novel cell subsets as well as elucidation of rarer cell types that do not separately cluster in the initial clustering (e.g. Mast cells, Lymphatic endothelial cells).

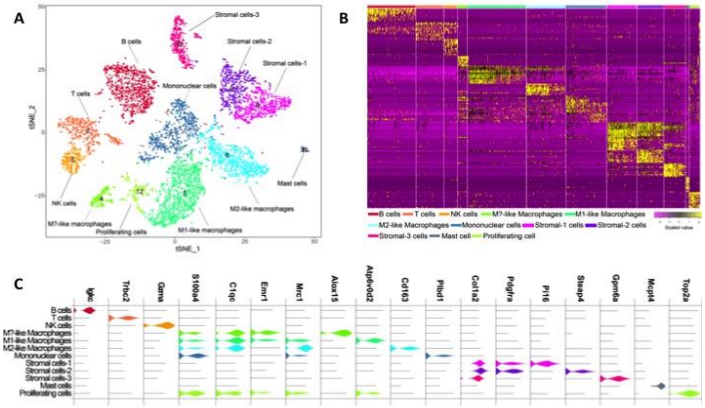
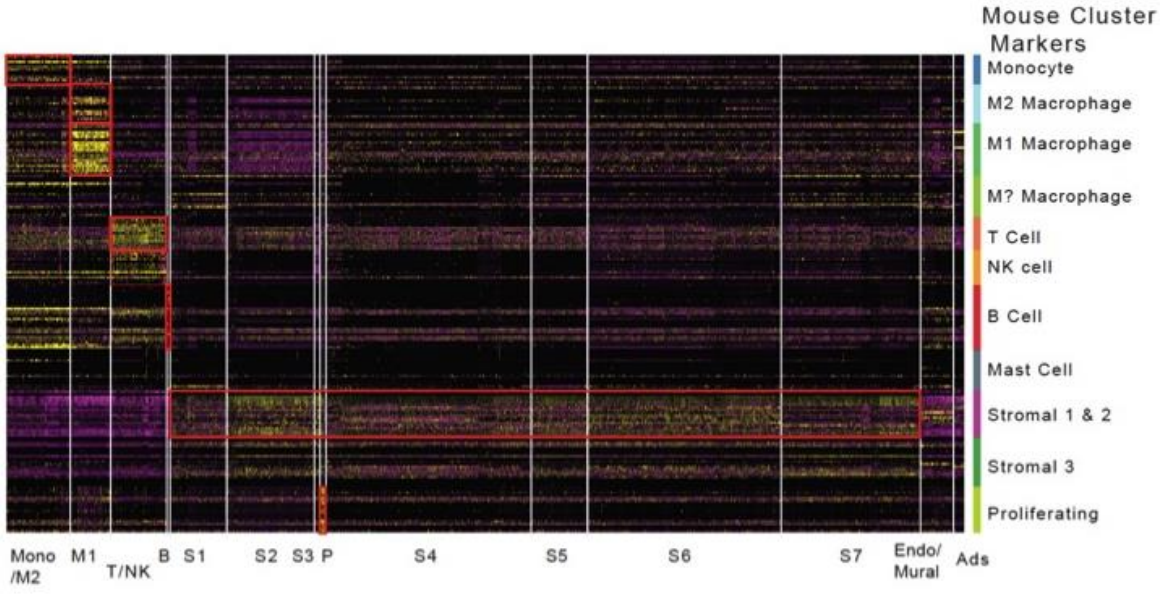


Fig. 3. scRNA-seq analysis of eWAT SVF from chow-fed male C57 mice. ***A***, ***B***, t-SNE plot and heatmap of SVF cells. ***C***, Unique markers exist for different cell sub-populations.

Similarly, we can find both well described as well as unexpected cell types in mouse data (Figure3) and can identify specific markers for each cell type. In a preliminary comparison of markers across species, we checked for expression of the human ortholog for mouse cell type markers (Figure 4), and found some markers and marker sets that commonly marked the analogous human cell type (eg M1 macrophages, T cells, proliferating cells), and others where top markers were not shared across species (Monocyte, M2 Macrophages, Mast Cells).



What opportunities for training and professional development has the project provided?
I take part in the Boston single-cell working group in part due to this project.

How were the results disseminated to communities of interest?
I have presented preliminary analyses in both internal group and division wide lab meetings.

What do you plan to do during the next reporting period to accomplish the goals?
We plan to finish adipose sample collection in the last period. We will collect samples from an additional 5 individuals with varying levels of insulin resistance. Additonally, we will collect as many paired omental and subcutaneous adipose samples from subjects having abdominal surgeries to examine depot-specific cell types and transcriptional profiles and finish the mouse experiments using paired inguinal and epididymal samples from HFD-fed mice.

We will continue to add new to existing data and perform iterative unbiased clustering analyses to determine transcriptionally distinct cell types (rarer cell types especially benefit from the additional data). Using this atlas, we will refine the markers for individual cell types and determine whether any cell populations correlate with any of the phenotypic parameters we collect (BMI, HOMA-IR, serum cholesterol triglyceride, CRP). We will also compare gene expression from individual cell types from lean vs. obese and insulin sensitive vs. insulin resistant subjects to determine how each individual cell type within adipose SVF responds transcriptionally to their metabolic phenotype. Lastly, we will begin cross-species comparisons both *in silico* by comparing identified cell type profiles and will begin validation experiments with FACs-based isolation of identified cell types.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
We have continued to make critical strides in developing a protocol to be able to assess individual cell type transcriptional profiles in adipose tissue SVF from human samples, including from size-limited omental biopsies during this reporting period. We are well on our way to finalizing an atlas of cell types within human adipose SVF, providing a delineation of the "parts" that make up adipose tissue as well as what are important genes that identify them. This should provide the field with molecular handles to better understand the and manipulate the function of specific cell types within this niche. Further, cell-type specific profiles can be used to better interpret specific cell types that may mediate genome wide association signals. By comparing the relative profiles of these cell types across individuals, we can also determine specific genes in specific cell types that correlate with metabolic characteristics such as obesity, diabetes, and cholesterol levels to better understand how specific genes and cell types modulate these metabolic characteristics.

What was the impact on other disciplines?
Nothing to Report

What was the impact on technology transfer?
Nothing to Report

What was the impact on society beyond science and technology?
Nothing to Report

CHANGES/PROBLEMS:

Changes in approach and reasons for change:
As discussed in more detail in last progress report, we have switched our technique for single cell RNA-seq profiling from SCRB-seq to Drop-seq for all experiments due to improvements in efficiency, cost, and feasibility. We have additionally added mouse experiments to better control for genetic and environmental effects seen in human data.

Delays:
As per last progress report, we encountered a delay in production phase due to our preliminary SCRB-seq trials not working and the set-up time involved in switching to Drop-seq. We have also encountered some recent delays in expected patient accrual of omental adipose tissue samples due to departure of a collaborating surgeon. We are recruiting multiple surgeons to replace this pipeline.

Changes that had a significant impact on expenditures:
As per last progress report, the change from SCRB-seq and Drop-seq techniques required moving funding from initial proposal of outsourcing library construction and sequencing costs to the Broad Institute to performing Drop-seq library construction and sequencing within our lab. This meant removing FACS core funding, purchasing of a new thermocycler in order to perform the library construction, and moving some of initial SCRB-seq costs to supporting the research assistant who is generating the libraries and sequencing them. As Drop-seq is more cost-efficient on a per cell basis, we have been able to increase the number of SVF cells we are assessing per subject to ~5K cells per person for at least 10 individuals (our initial estimate with SCRB-seq was to profile 21,000 cells total).

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Significant changes in use or care of human subjects:
none, IRB renewed since last progress report. Approval Date: 12/14/2016, Expiration Date: 12/13/2017

Significant changes in use or care of vertebrate animals:
We are using existing animal protocol from Dr. Rosen (056-2017) for feeding chow and HFD and harvesting tissue.

Significant changes in use of biohazards and/or select agents:
N/A

PRODUCTS:

Publications, conference papers, and presentations

Journal publications: Nothing to Report. (Manuscript based on pilot arcuate hypothalamus data was accepted at *Nature Neuroscience* (Campbell JN, Macosko EZ, Fenselau H, Pers TH, Lyubetskaya A, Tenen D, Goldman M, Verstegen AMJ, Resch JM, McCarroll SA, Rosen ED, Lowell, BB, Tsai LT. A molecular census of arcuate hypothalamus and median eminence cell types *Nat Neurosci*. 2017; 20(3):484-496. PMCID: PMC5323293).

Books or other non-periodical, one-time publications: Nothing to Report

Other publications, conference papers, and presentations:

1) "A Transcriptomic Atlas of Arcuate Cell Types", Boston Single Cell Working Group, (Local meeting)
2) 03/17 A Molecular Census of Arcuate Hypothalamus and Median Eminence Cell Types. Medical and Population Genetics Program Meeting, Broad Institute (Local Meeting)

Website(s) or other Internet site(s):
Nothing to Report

Technologies or techniques:
Nothing to Report

Inventions, patent applications, and/or licenses:
Nothing to Report

Other Products:
Database of human SVF cell types, mouse arcuate cell types. Upon publication, these transcriptional data will be provided to the public via NIH's GEO database.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Linus Tsai, MD, PhD
Project Role:	PI
Researcher Identifier	ORCID ID 0000-0002-0134-6949
Nearest person month worked:	2.3
Contribution to Project:	Performing patient recruitment, sample collection and processing, Dropseq, and directing library construction, sequencing and analysis
Funding Support:	Boston Area Diabetes Endocrinology Research Center Pilot and Feasibility Grant (NIH 2P30DK057521-16), Boston Nutrition and Obesity Research Center Core Grant (5 R01 DK 087092-05)

Name:	Anna Lyubetskaya, PhD
Project Role:	Computational Biologist
Researcher Identifier	eracommons: alyubets
Nearest person month worked:	0.6
Contribution to Project:	Dr. Lyubetskaya has performed computational analysis pipeline and developed methods for analyzing single cell transcriptional data
Funding Support:	

Name:	Danielle Tenen
Project Role:	Research Assistant
Researcher Identifier	ORCID ID 0000-0001-6890-5488
Nearest person month worked:	4.3
Contribution to Project:	Performing sample processing, Dropseq library construction, sequencing
Funding Support:	Boston Area Diabetes Endocrinology Research Center Pilot and Feasibility Grant (NIH 2P30DK057521-16), Boston Nutrition and Obesity Research Center Core Grant (5 R01 DK 087092-05)

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Yes, for Linus Tsai, one new grant has been funded.
4/1/2017-3/31/2022 TGFbeta-mediated Transcriptional Reprogramming of Mature Adipocytes in Obesity
NIH/NIDDK R01 DK113669 (Rosen)
Co-Investigator ($320,000 DC/$227,021 IDC)
The goal of this project is to identify early mechanisms underlying adipose tissue fibrosis arising within the mature adipocyte, focusing on TGFbeta-mediated transcription through Smad3 and SRF.

What other organizations were involved as partners?
Organization Name: Broad Institute
Location of Organization: Cambridge, MA
Partner's contribution to the project: Facilities (Provide computing infrastructure for analyses)

SPECIAL REPORTING REQUIREMENTS
COLLABORATIVE AWARDS: N/A
QUAD CHARTS: N/A

APPENDICES:
Nothing to report